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Regulation of Pyruvate Carboxylase by 1,25(OH)2D in MCF10 Breast Epithelial Cells

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REGULATION OF PYRUVATE CARBOXYLASE BY 1,25$(OH)_2$D IN MCF10 BREAST EPITHELIAL CELLS

by

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Literature Review & Rationale

Breast cancer is the second most common cancer in women. According to the Centers for Disease Control and Prevention, 211,731 women were diagnosed with breast cancer and 40,676 women died from it in the United States in 2009 (US Cancer Statistics). Breast cancer tumors go through stages of development termed carcinogenesis. Carcinogenesis begins with tumor initiation, followed by tumor promotion, malignant conversion, and lastly tumor progression (Weston, 2000). Tumor initiation starts with genetic mutations, most often associated with activation of proto-oncogenes or loss of tumor-suppressor genes. This process is referred to as multistage carcinogenesis.

Oncogenes are normal cell genes that control cell proliferation, differentiation, and apoptosis. When altered or mutated, however, they can cause uncontrolled cell proliferation and decreased apoptosis resulting in breast cell tumorigenesis (Weston, 2000). The Harvey-*ras* oncogene is present in many breast cancer tumors. Both cellular and animal models have shown that transfection of mutated, active forms of *ras* oncogene into untransformed cells results in invasive and metastatic phenotype (Bondy, 1985; Bradley, 1986).

Vitamin D is metabolized in many tissues and its circulating form in the body, 25-hydroxyvitamin D (25OHD), is generated in the liver by the enzyme 25-hydroxylase. 25OHD is further hydroxylated by 1α-hydroxlase enzyme in several tissues to its active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Lips, 2006). The active form of vitamin D, $1,25(OH)_2D$, acts as a ligand to the vitamin D receptor (VDR). Active form of vitamin D, $1,25(OH)_2D$, binds to the nVDR which heterodimerizes with the retinoid X receptor (RXR) and then translocates to

the nucleus of target cells, where it binds to vitamin D response elements on DNA (Haussler, 2006). This is significant because $1,25(OH)_2D$-induced gene transcription has been shown to regulate many cellular processes involved with carcinogenesis such as proliferation, differentiation, and angiogenesis.

Recent research on the United States population found the overall prevalence rate of vitamin D deficiency to be 41.6%. (Forrest, 2010). Vitamin D can be obtained through supplements or through foods like fish liver oils, flesh of fatty fish, and fortified milk and cereals (American Dietetics Association). Most foods do not contain vitamin D naturally, so the majority of vitamin D is obtained through UV and sunlight exposure. Ultraviolet radiation (UV-B) is required for conversion of 7-dehydrocholesterol into vitamin D in the skin (Giovannucci, 2005). Epidemiological studies have shown a higher incidence for breast cancer in areas with higher latitude and lower sunlight, which is associated with a low vitamin D synthesis in the skin (Garland, 2006). In addition, studies in which vitamin D status was measured showed that low serum $1,25(OH)_2D$ concentrations were associated with increased breast cancer risk and disease progression (Welsh, 2004). As stated previously, $1,25(OH)_2D$-induced gene transcription has been shown to regulate many cellular processes involved with carcinogenesis through multiple cell signaling pathways that regulate proliferation, differentiation, apoptosis, and most likely angiogenesis, characteristics of cancer cells (Narvaez, 2001). This is interesting particularly as studies have shown that cells transfected with the Harvey-*ras* oncogene (*ras* cells) demonstrate significant reduction in $1,25(OH)_2D$-induced transactivation of the nuclear vitamin D receptor (nVDR) compared with nontransfected cells (Taber, 2009).

Recent work in our laboratory has shown that $1,25(OH)_2D$ affects breast cancer cells' energy metabolism, as well, by reducing glycolysis, lactate synthesis, and glucose flux into the TCA cycle. In contrast to normal differentiated cells, which rely primarily on mitochondrial oxidative phosphorylation to generate the energy needed for cellular processes, most cancer cells rely on aerobic glycolysis (Vander Heiden, 2009.). This phenomenon is termed "the Warburg effect". More specifically, "the Warburg effect" explains that cancer cells tend to "ferment" glucose into lactate, even in the presence of oxygen, which is unlike normal cell tissues (Vander Heiden, 2009). In addition to the preferential metabolism of fermentation in cancer cells, evidence shows that tumor cells demonstrate significantly increased rates of glucose uptake compared with a normal cell (Locasale, 2011). Aerobic glycolysis is an inefficient way to generate ATP, so the advantage it confers for cancer cells is unclear. However, it is proposed that the increase in glycolysis provides carbon backbones for the synthesis of compounds needed for growth, such as nucleotides. Why cancer cells utilize fermentative metabolism is complex and remains a hotly disputed topic (Locasale, 2011).

There are a number of proposed reasons for why cancer cells utilize an energy cycle that produces only 2 ATPs per molecule of glucose, opposed to utilizing the energy cycle of a normal cell producing 38 ATPs. One explanation reflects on the idea that inefficient ATP production is a problem only when resources are scarce for the cell (Van Heiden, 2009). Proliferating cells, like active cancer cells, are exposed to continual supplies of glucose and other nutrients in circulating blood. There is evidence that ATP may never be limiting in proliferating mammalian cells (Van Heiden, 2009).

Proliferating cells may likely have metabolic requirements that require components like amino acids, nucleotides, and lipids, in order to generate biomass (Van Heiden, 2009). In proliferating cells, instead of a pressure to optimize metabolism for ATP yield, a selective pressure for rate of metabolism does exist. To survive, the cell must maximize their rate of anabolic growth. Cells that convert glucose into biomass most efficiently will proliferate fastest (Vander Heiden, 2009).

As mentioned previously, evidence from our laboratory shows that the activity of the aerobic glycolysis noted in the Warburg effect can be reduced by treatment of $1,25(OH)_2D$ in early carcinogenesis. **Figure 1** shows results from experiments conducted in our laboratory using untransformed MCF10A breast epithelial cells and H-*ras* oncogene transfected breast epithelial cells. The data showed a decreased amount of glucose influx across the cell membrane of cells treated with $1,25(OH)_2D$ compared to the vehicle. Also, the amount of intracellular lactate was significantly lower in MCF10A-*ras* cells treated with $1,25(OH)_2D$ compared to the vehicle. In addition, the flux of glucose to acetyl-coA and oxaloacetate levels was significantly lower in the cells treated with $1,25(OH)_2D$ compared to the vehicle. These results provide evidence that $1,25(OH)_2D$ regulates glucose metabolism in MCF10A and MCF10A-*ras* cells breast epithelial cells.

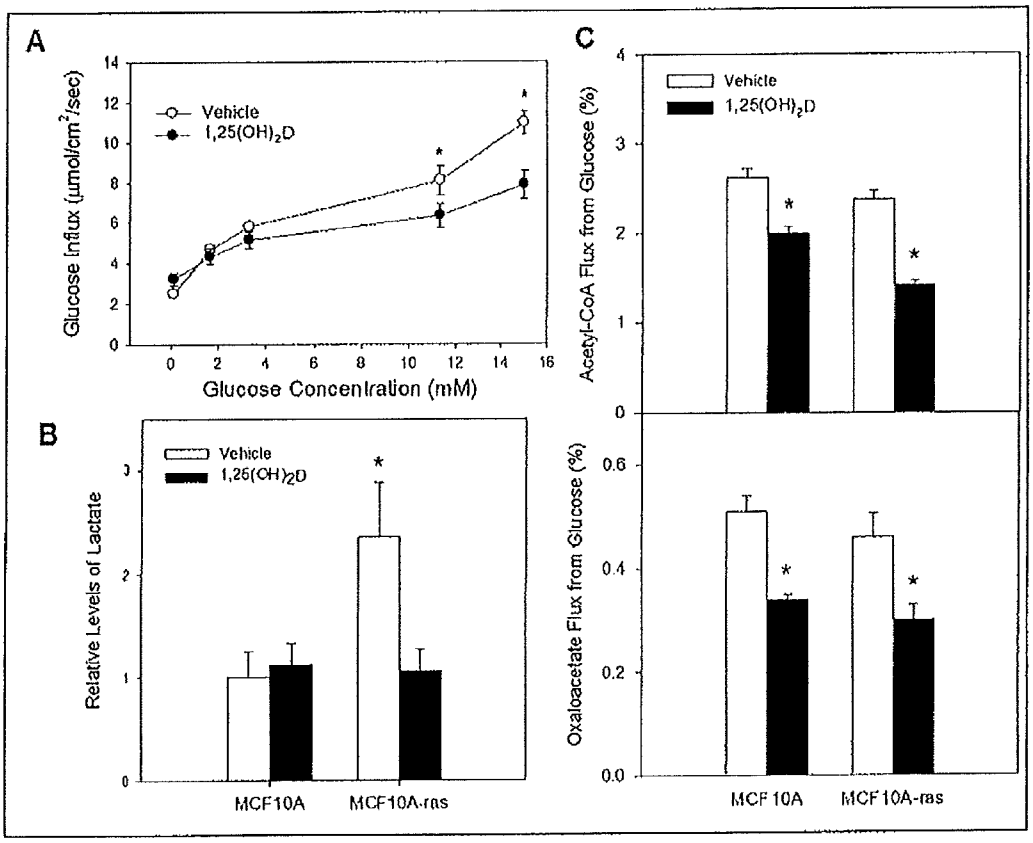

Figure 1. $1,25(OH)_2D$ regulation of glucose uptake, lactate production and glucose contribution into TCA cycle. MCF10A and MCF10A-*ras* cells were treated with vehicle or $1,25(OH)_2D$ for 4 days. (A) Glucose influx at the cell membrane in response to increasing doses of added glucose in the media in MCF10A-*ras* cells; (B) Intracellular levels of lactate relative to vehicle treatment in each cell type; (C) Flux contributions of $^{13}C_6$-labeled glucose to acetyl-CoA and oxaloacetate shown in percent metabolite flux from glucose. * Indicates a significant difference between vehicle and $1,25(OH)_2D$ treatments within the same cell type ($n=4$, $P<0.05$).

Figure 2 shows a summary of the effect of $1,25(OH)_2D$ seen on several aspects of the energy cycle based on the evidence from our laboratory. In cells treated with vitamin D, a reduced uptake of glucose was observed, decreased production of pyruvate and lactate, and decreased production of TCA cycle intermediates.

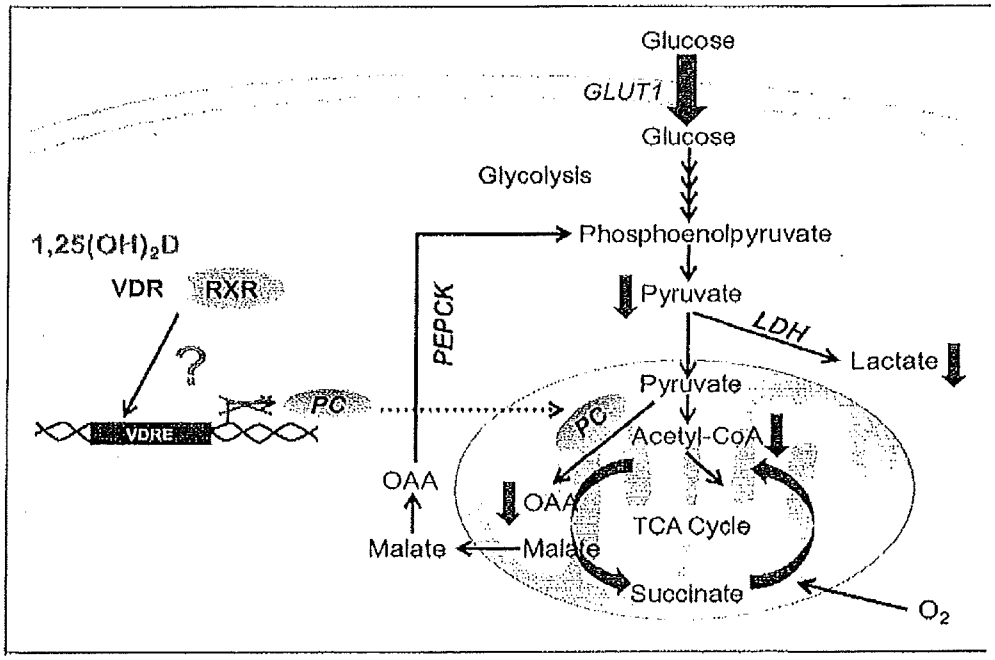

Figure 2. Impact of vitamin D on glucose metabolism in carcinogenesis
$1,25(OH)_2D$ reduces glucose uptake and the flux of glucose into glycolytic intermediates and lactate, as well as the TCA cycle intermediates (shown by red arrows).

PC: pyruvate carboxylase
RXR: retinoid X receptor
GLUT1: glucose transporter 1
LDH: lactate dehydrogenase
VDR: vitamin D receptor
OAA: oxaloacetate
PEPCK: phosphoenolpyruvate carboxykinase

The mechanism by which $1,25(OH)_2D$ influences these changes is not known, but our laboratory has investigated the role of pyruvate carboxylase in $1,25(OH)_2D$ regulation. Pyruvate carboxylase is an enzyme expressed in gluconeogenic tissues like the liver, as well as adipose tissue and lactating mammary glands. It uses the pyruvate created in the last step of glycolysis to

create oxaloacetate. Pyruvate carboxylase is the enzyme used in the first step in gluconeogenesis, a process used to re-create glucose from pyruvate. Our preliminary laboratory results shown in **Figure 3** demonstrate that $1,25(OH)_2D$ reduces the mRNA expression of pyruvate carboxylase in a model of very early, initiation phase in cancer, suggesting that they may be the primary targets that mediate the impact of $1,25(OH)_2D$ on energy metabolism. The regulation of pyruvate carboxylase by $1,25(OH)_2D$ in models of early, but more progressed cells lines has not been determined. Further research on $1,25(OH)_2D$'s effects on pyruvate carboxylase activity may provide the mechanistic basis for the regulation of energy metabolism during early carcinogenesis.

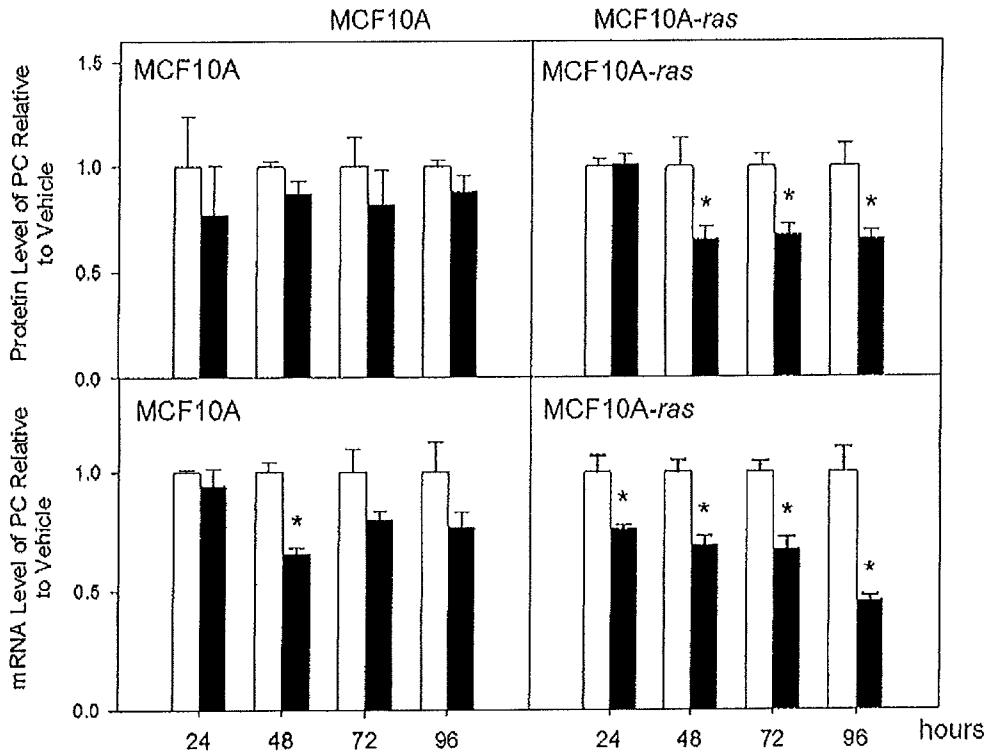

Figure 3. $1,25(OH)_2D$ regulation of pyruvate carboxylase (PC) expression. Protein (upper panel) and mRNA (lower panel) expression of PC in MCF10A and MCF10A-*ras* cells treated with vehicle (open bars) or $1,25(OH)_2D$ (black bars) for indicated times. * Indicates a significant difference between vehicle and $1,25(OH)_2D$ treatments

Purpose

The purpose of this study is to determine the impact of $1,25(OH)_2D$ on enzyme pyruvate carboxylase (PC) protein expression and mRNA expression in MCF10CA1h and MCF10CA1a breast epithelial cells.

Study Design & Methods

Cell Culture

The MCF10CA1h and MCF10CA1a human breast epithelial cells were used in the experiments. One of the hurdles in cancer prevention research is the few models available for studying cancer progression. In order to identify cancer preventive agents, it is critical to use model systems that include untransformed cells and well characterized models from early pre-cancer to malignant and highly metastatic cells. For this reason, we use the MCF10 model of breast cancer progression in which progressively more malignant mammary epithelial cell lines were obtained by transformation and subsequent xenograft cycling as tumors in nude mice. All cells in the model are derived from the spontaneously immortalized MCF10A cell line, thus have the same original genetic background. The model includes the untransformed MCF10A cells; the

Harvey-*ras* oncogene transfected MCF10A-*ras* cells, which are mildly hyperplastic *in vivo*; and the tumorigenic MCF10CA1h cells that are derived from MCF10A-*ras* cells through serial in vivo passage which are non-metastatic carcinoma in situ (Richardson, 2008; Santner, 2001). The MCF10CA1h and MCF10C1a cell lines used in this study are both derived from MCF10A-ras xenografts, and form well-differentiated and poorly differentiated malignant tumors in their respective models (Kadota, 2010). Thus we consider the MCF10 cell system to be an appropriate model of mammary carcinogenesis.

These cells were maintained in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F12(1x)), containing 5% horse serum and 100 units/ml of penicillin in a humidified environment at 37 C. Cells were plated at a density of 350,000 cells per 60mm culture dish and treated with vehicle (ethanol) or 10 nM of $1,25(OH)_2D$ for 48 hours.

Methods

Total RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. Reverse transcription of total RNA was completed by using MMLV reverse transcriptase (Promega, Madison, WI). Quantifications of cDNA were performed using Brilliant II SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA). The mRNA abundance of PC was determined from the threshold cycle (Ct) value. The mRNA expression was then compared to the 18s expression and results were expressed as arbitrary units.

Bicinchoninic acid protein (BCA) assay was used to determine protein concentration from cell lysate samples and then total level of protein was determined by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Kaleidoscope (Bio-Rad, Hercules, CA) and Biotin (Cell Signaling Technology, Beverly, MA) markers were used as instructed by the manufacturer. The electrophoresis was run with a 7.5% Tris-HCL gel transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). A 1:2000 dilution for the PC primary antibody (anti-rabbit) (Santa Cruz Biotechnology, Santa Cruz, CA) was used. Actin protein (42 kDa) was used as a standard marker with a 1:2000 dilution for the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), a 1:2000 (anti-Rb) and 1:2000 (anti-biotin) dilution for secondary antibody. Lumiglo Reagent A and Peroxide Reagent B (Cell Signaling Technology, Beverly, MA) were used for blotting and diluted in accordance with the manufacturer's instructions. Quantifications of the westerns were done using UN-SCAN-IT Program.

Statistical Analysis

Results were compared using t-tests or by analysis of variance (ANOVA). A p-value of less that 0.05 is defined as statistically significant.

Results

Impact of 1,25(OH)$_2$D on PC protein expression in MCF10CA1h and MCF10CA1a cells after 24 hour treatment.

In order to determine if 1,25(OH)$_2$D regulates pyruvate carboxylase in breast epithelial cells in a more advanced stage of carcinogenesis, protein expression of pyruvate carboxylase

following 24 hour treatment was determined in MCF10CA1h and MCF10CA1a cells. In the MCF10CA1h breast epithelial cells, the addition of 10nM of $1,25(OH)_2D$ did not significantly impact the expression of PC protein (P=.32) relative to the vehicle (EtOH) (**Fig. 4**). In the MCF10CA1a breast epithelial cell line, the addition of 10 nM of $1,25(OH)_2D$ did significantly reduce the protein expression of PC relative to vehicle (P=.04) (**Fig. 4**).

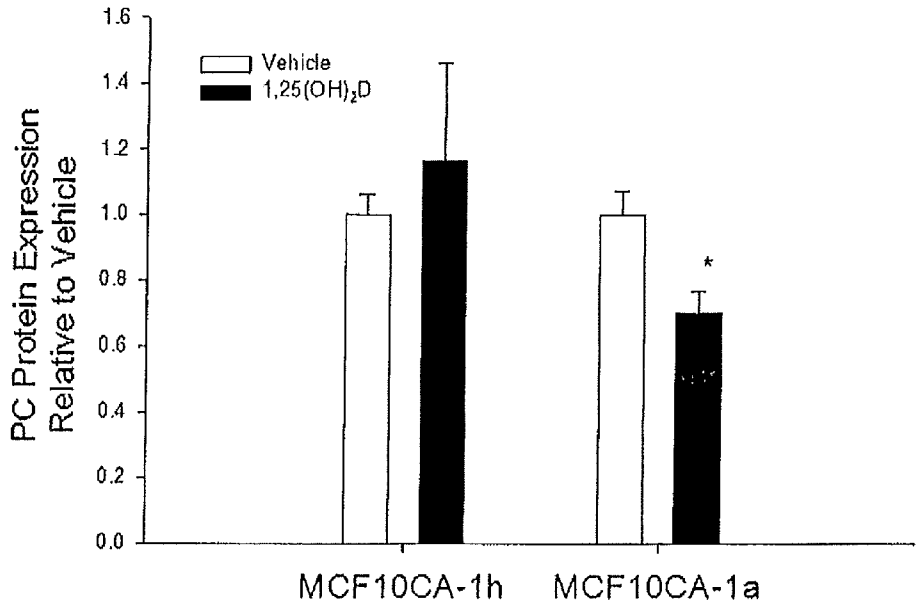

Figure 4: **$1,25(OH)_2D$ Regulation of PC protein,** MCF10CA1h and MCF10CA1a cells were treated with either vehicle or 10nM of $1,25(OH)_2D$ for 24 hours, and harvested for determination of PC protein expression by Western Blots. Bars with asterisks (*) indicate significance (P=<0.05) as assessed by t-tests.

Impact of $1,25(OH)_2D$ on PC mRNA expression in MCF10CA1h and MCF10CA1a cells after 48 hour treatment.

Again, in order to determine if $1,25(OH)_2D$ regulates PC in breast epithelial cells in a more advanced stage of carcinogenesis, mRNA expression of PC following 48 hour treatment was determined in MCF10CA1h and MCF10CA1a cells. This also contributes to understanding the mechanism by which $1,25(OH)_2D$ regulates PC as changes in mRNA would suggest transcriptional control. In the MCF10CA1h breast epithelial cells, the addition of 10nM of $1,25(OH)_2D$ did not significantly impact the expression of PC mRNA (P=.31) relative to the vehicle (EtOH) **(Fig. 5).** In the MCF10CA1a breast epithelial cell line, the addition of 10nM of $1,25(OH)_2D$ significantly reduced the expression of PC mRNA abundance relative to vehicle (P=.00006) **(Fig. 5).**

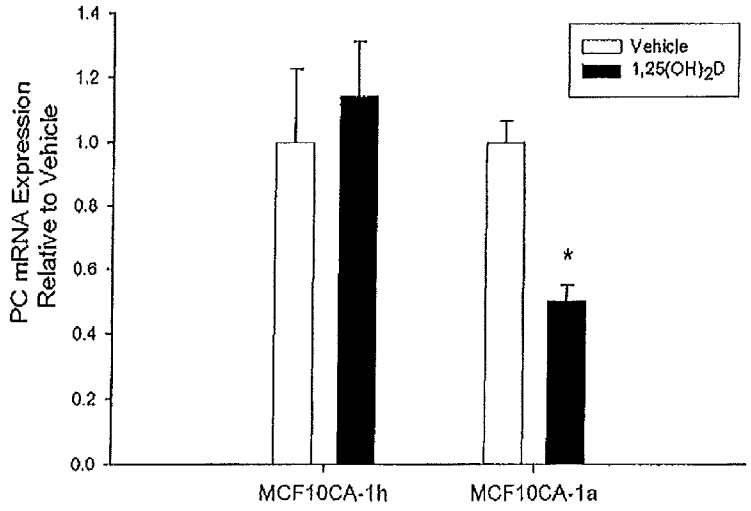

Figure 5: **$1,25(OH)_2D$ Regulation of PC mRNA,** MCF10CA1h and MCF10CA1a cells were treated with either vehicle or 10nM of $1,25(OH)_2D$ for 48 hours, and harvested for determination of PC mRNA expression by RT-PCR. Bars with asterisks (*) indicate significance (P=<0.05) as assessed by t-tests.

Impact of 1,25(OH)$_2$D on PC protein expression in MCF10CA1h and MCF10CA1a cells after 48 hour treatment.

Protein expression of pyruvate carboxylase following a 48 hour treatment was determined in MCF10CA1h and MCF10CA1a cells. In the MCF10CA1h breast epithelial cells, the addition of 10nM of 1,25(OH)$_2$D did not significantly impact the expression of PC (P=0.46) relative to the vehicle (EtOH) **(Fig. 6).** In the MCF10CA1a breast epithelial cell line, the addition of 10nM of 1,25(OH)$_2$D significantly reduced the expression of PC protein level (P=0.03) level relative to the vehicle (EtOH) **(Fig. 6).**

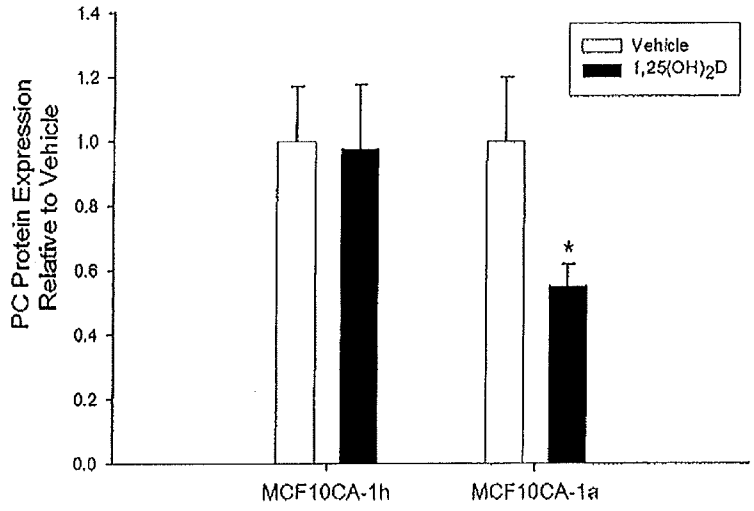

Figure 6: **1,25(OH)$_2$D Regulation of PC protein,** MCF10CA1h and MCF10CA1a cells were treated with either vehicle or 10nM of 1,25(OH)$_2$D for 48 hours, and harvested for determination of PC protein expression by Western Blots. Bars with asterisks (*) indicate significance (P=<0.05) as assessed by t-tests.

Discussion

Vitamin D is associated with decreased risk of breast cancer, but by what mechanism is unclear. Our previous studies have shown that $1,25(OH)_2D$ reduces glucose uptake and the flux of glucose into glycolytic intermediates and lactate, as well as the TCA cycle intermediates, in MCF10A and MCF10A-*ras* cells. In this study, we show decreasing expression of enzyme PC in breast cancer cell models may be the mechanism by which $1,25(OH)_2D$ regulates the energy metabolism of breast cancer cells. The mRNA levels and protein expression of PC was decreased relative to the vehicle in MCF10CA1a cell line ($P<0.05$). However, the mRNA levels and protein expression of PC was unaffected by $1,25(OH)_2D$ relative to the vehicle in MCF10CA1h cell line. These results suggest that PC plays a role in $1,25(OH)_2D$ regulation of the energy metabolism in MCF10CA1a breast cells, but not in MCF10CA1h cells.

Our results indicate that pyruvate carboxylase in MCF10CA1h cells does not respond to $1,25(OH)_2D$. It is known that MCF10CA1h cells have reduced vitamin D receptor activity compared to other cell lines, however, we do not know why there is no response in these cells. A possible explanation is that the vitamin D response element (VDRE) could be inhibited in MCF10CA1h cells through an unknown mechanism.

The $1,25(OH)_2D$ mediated decrease in pyruvate carboxylase protein expression in MCF10CA1a cells is consistent with the decrease seen in less progressed breast cell lines (MCF10A-*ras*) in our previous studies. These results imply that there is a decrease in the flux of glucose into the TCA cycle in breast cancer cells. The results further indicate that $1,25(OH)_2D$ may more effectively impact the process of carcinogenesis in more progressed cell models such as MCF10CA1a cells in addition to the earlier cells tested in our previous studies. This is

significant because determining a target by which vitamin D can regulate more progressed, aggressive, breast cells derived from malignant tumors is important in making steps towards developing recommendations for decreasing metastasis and cancer cell proliferation.

Additional studies are necessary to determine if pyruvate carboxylase plays a role in $1,25(OH)_2D$ mediated changes in energy metabolism during tumor progression, and if it has an impact in suppressing tumor progression.

Conclusion

In MCF10CA1h breast epithelial cells, treated with $1,25(OH)_2D$ for both 24 and 48 hours, the mRNA levels and protein expression of pyruvate carboxylase showed no significant change relative to the vehicle control. This indicates that expression of pyruvate carboxylase in MCF10CA1h cells does not respond to $1,25(OH)_2D$. In MCF10CA1a breast epithelial cells, treated with $1,25(OH)_2D$ for 24 hours, the protein expression of pyruvate carboxylase significantly decreased. In MCF10CA1a breast epithelial cells, treated with $1,25(OH)_2D$ for 48 hours, the mRNA levels and protein expression of pyruvate carboxylase were significantly decreased. This indicates that pyruvate carboxylase is regulated by $1,25(OH)_2D$ in MCF10CA1a cells. It also suggests that $1,25(OH)_2D$ may more effectively impact the process of carcinogenesis in more progressed cancer cell models such as MCF10CA1a cells.

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